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CELL VOLUME REGULATION BY MOLLUSCAN ERYTHROCYTES DURING HYPOOSMOTIC STRESS: Ca^{2+} EFFECTS ON IONIC AND ORGANIC OSMOLYTE EFFLUXES

LAURENS H. SMITH JR.[1] AND SIDNEY K. PIERCE[1,2]

[1]*Department of Zoology, University of Maryland, College Park, Maryland 20742 and* [2]*Center of Marine Biotechnology, 600 East Lombard St., Baltimore, Maryland 21202*

ABSTRACT

The role of Ca^{2+} in volume regulation remains obscure. Before it can be investigated, however, the time courses of osmolyte and cell volume regulation and the effect of Ca^{2+} must be simultaneously specified in a suitable cell type. We have tested the red blood cells of *Noetia ponderosa* in that context. Our results show that the regulation of cell volume of the erythrocytes following hypoosmotic stress has two components. The first is an efflux of intracellular K^+ and Cl^- (but not Na^+) that begins immediately with the onset of hypoosmotic exposure. The second component, an efflux of taurine, follows the first, but only after many minutes. In addition, clam erythrocyte volume regulation is dependent on external [Ca^{2+}]. Volume recovery is potentiated in hypoosmotic media containing elevated Ca^{2+} levels. Taurine efflux from clam erythrocytes in hypoosmotic conditions is reduced in Ca^{2+}-free media and potentiated in high Ca^{2+} media. In contrast, the effluxes of K^+ and Cl^- are not sensitive to extracellular Ca^{2+} levels in either isosmotic or hypoosmotic media. Thus, the effluxes of ionic and organic osmolytes from these cells are controlled by mechanisms that differ in response time and Ca^{2+} sensitivity. These results suggest that the clam cells have an unexceptional volume regulatory mechanism and should therefore make an excellent model with which to study the role of Ca^{2+} in that process.

INTRODUCTION

Cell volume in response to hypoosmotic stress is controlled by regulation of the amount of intracellular osmolytes. Cell volume increases rapidly when water enters in response to hypoosmotic exposure, but then decreases as an efflux of intracellular osmolytes removes osmotically obligated water.

The cells of marine invertebrates use intracellular free amino acids (FAA, for review see Pierce, 1982) or other small nitrogenous compounds (proline betaine, Pierce *et al.*, 1984; glycine betaine, Warren and Pierce, 1982) as osmolytes. In some invertebrate cell types, an intracellular inorganic component to cell volume regulation has been found in addition to the organic osmolytes (*Limulus* myocardium, Warren and Pierce, 1982; *Glycera* coelomocytes, Costa and Pierce, 1983; *Noetia* red blood cells, Smith and Pierce, 1983; *Cancer* leg muscle, Moran and Pierce, 1984). The ions (Na^+, or K^+, and Cl^-) leave the cell as soon as the hypoosmotic stress begins, and the organic efflux follows at a slower rate.

In addition to the above characteristics, cell volume regulation in response to a hypoosmotic stress seems to be affected by external Ca^{2+} concentrations (for a review see Pierce and Amende, 1981; Pierce, 1982). Generally, when Ca^{2+} is reduced or

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Abbreviations: FAA, free amino acids; ASW, artificial seawater; EGTA, ethyleneglycol-bis-(β-amino ethyl ether) N,N′-tetraacetic acid.

omitted from the medium, cell volume regulation is either blocked or reduced. Free $[Ca^{2+}]_i$ is too low to exert a substantial osmotic effect. Thus, the role played by Ca^{2+} in the volume recovery must be either to activate or to regulate the osmolyte efflux mechanism. Since understanding that regulatory mechanism is a central issue in cell volume regulation, we have begun to investigate the Ca^{2+} effects more closely.

Unfortunately, the characteristics of the volume regulatory mechanism have not been examined in sufficient detail in any single cell type to serve as a point of departure. Therefore we have selected a single cell type, the red blood cell of the bivalve, *Noetia ponderosa,* and have described the entire process of osmolyte regulation during the volume response to a hypoosmotic stress on this system. In addition, we have determined the effect of $[Ca^{2+}]_0$ on each step of the process. Our results provide the first simultaneous chronology of all the events (ion, amino acid, and volume changes) that constitute cell volume recovery from a hypoosmotic stress, including the effects of Ca^{2+} on that chronology. In addition, the similarity of our results to those from other cells indicates that the *Noetia* cells are a useful model with which to study the effects of Ca^{2+} further. A preliminary report of this study has been published earlier (Smith and Pierce, 1983).

Materials and Methods

Preparation of erythrocytes

N. ponderosa were collected by commercial dredge and maintained as described by Amende and Pierce (1980a).

Blood was collected from the clams at room temperature by forcing the valves open and slashing the mantle. The blood was diluted with artificial seawater (ASW, 935 mosm) made according to Amende and Pierce (1980) and buffered to pH 7.4 with 5 m*M* MOPS. The blood was then filtered through polyester wool. The erythrocytes were centrifuged twice at $500 \times g$; the supernatants were discarded and the cells were resuspended in ASW between the spins. After the second centrifugation, the cells were resuspended in ASW and centrifuged at $3000 \times g$, and the top layer of reproductive and amoebocytic cells was removed by aspiration. The erythrocyte pellet was washed twice more and the erythrocytes were then resuspended in approximately 1 ml of ASW for further use.

Measurement of cell volume regulation

Cell volumes of *Noetia* erythrocytes were measured in isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW, or in iso- or hypoosmotic media containing increased Ca^{2+} or no Ca^{2+} (Table I). Hypoosmotic ASW was made by diluting isosmotic ASW with glass distilled water. MOPS was always kept at 5 m*M*, and the pH at 7.4. The osmotic concentrations of the solutions containing 150 m*M* Ca^{2+} were matched to the control solutions by reducing the NaCl content. The high Ca^{2+} isosmotic solution was made fresh just before use as follows: first, the chloride salts of Na^+, K^+, and Mg^{2+} were dissolved in distilled water in the concentrations indicated in Table I. $CaCl_2$ was then added to the mixture to produce a final concentration of 150 m*M*. Finally, predissolved solutions of Mg_2SO_4 and $NaHCO_3$ were added very slowly with stirring to produce the concentrations of the ions listed in Table I.

NaCl content was increased to account for the osmotic deficit produced by Ca^{2+} removal in the Ca^{2+}-free ASW. This last solution also contained 1 m*M* EGTA. The osmotic concentrations of all solutions were determined before use with a vapor pressure osmometer (Wescor, model 5100C).

Approximately 4.0×10^6 washed cells were suspended in each experimental me-

TABLE I

Ionic composition (in mmoles/liter) of isosmotic artificial seawaters used as experimental media

Ion	Control	Ca^{2+}-Free	High Ca^{2+}
NaCl	389.0	400.1	249.6
$MgCl_2$	24.4	24.4	24.4
$CaCl_2$	9.7	0	150.1
KCl	9.7	9.7	9.7
$MgSO_4$	27.0	27.0	27.0
$NaHCO_3$	2.3	2.3	2.3
EGTA	—	1.0	—
MOPS	5.0	5.0	5.0

dium and the distribution of cell volumes in a 10,000 cell aliquot was measured after 5, 10, 20, 60, and 120 min of exposure to the media using an electronic cell sizer (Coulter Counter Model ZB) and Coulter Channelyzer (Coulter Electronics Inc., Hialeah, FL) interfaced with an Apple II+ computer. The time course of erythrocyte volume change was measured in both high Ca^{2+} hypoosmotic and Ca^{2+}-free hypoosmotic ASWs following a 30 min preincubation of the cells in 935 mosm ASW containing high Ca^{2+} and no Ca^{2+}, respectively.

Mean cell volumes from a given experiment were expressed as a percent of the mean cell volume at zero time. The differences in the percent initial cell volume in experimental media at all sampling times were tested using analysis of variance and the Student-Newman Keuls multiple range test (Steele and Torrie, 1960). $P \leq 0.05$ was considered significant.

Taurine efflux measurements

The amino acid efflux from *Noetia* erythrocytes exposed to hypoosmotic media consists primarily of taurine (Amende and Pierce, 1980). In addition, our preliminary experiments showed that when a treatment altered the amino acid efflux, the release of each amino acid was changed by the same proportion; the effluxes of the amino acids were not altered differentially. In particular, taurine always accounted for 60–70% of the total FAA efflux regardless of the magnitude of the FAA efflux. Therefore, we used taurine to represent amino acid efflux.

Clam erythrocytes were removed from the animal and prepared as described above. The cells were then placed into one of the experimental media. At appropriate intervals, aliquots containing 4–7 $\times$ 10^6 cells were removed from the experimental cell suspension and centrifuged at 5000 $\times$ g for 10 min. The taurine content of the supernatant was then determined as follows. An equal volume of 80% ethanol was added to the supernatant and the solution was placed into a boiling water bath for 20 min. Precipitated protein was removed by centrifugation at 25,000 $\times$ g for 20 min. The supernatant was then lyophilized, the residue taken up in 0.2 N lithium citrate buffer, pH 2.2, and the taurine content measured with an amino acid analyzer (JEOL JLC-6AH). The cell number in three 15–20 μl aliquots from each cell suspension was counted three times with the Coulter Counter and averaged to express the taurine levels in terms of cell number.

Significant differences in taurine efflux were determined by analysis of variance and the Student-Newman Kuels multiple range test.

Intracellular K^+, Na^+, and Cl^-

Erythrocytes were isolated as described above, and 10–20 $\times$ 10^6 cells were suspended in isosmotic media. Six 0.2 ml aliquots (approximately 1–8 $\times$ 10^6 cells) were then removed for measurement of intracellular K^+, Na^+, or Cl^- at zero time. The rest of the isosmotic suspension was divided in half and both halves centrifuged at 3000 $\times$ *g* for 5 min. The supernatants were aspirated and the cells resuspended in either isosmotic or hypoosmotic media and sampled at intervals. At each sampling time, triplicate 0.2 ml aliquots (approximately 1–8 $\times$ 10^6 cells) were removed from the experimental cell suspension for the measurement of intracellular K^+, Na^+, or Cl^-.

Intracellular K^+ was measured by the method of Costa and Pierce (1983). The 0.2 ml aliquot of cell suspension was layered onto 0.1 ml of silicone oil (Wm. F. Nye, Inc., New Bedford, MA 02742, USA) which had been layered over 0.1 ml of 25% glycerol. This preparation was then centrifuged at 3000 $\times$ *g* for 3 min (Beckman Microfuge II) which pelleted the cells through the oil into the glycerol where they lysed. The experimental medium remained on top of the oil. After the medium and oil layers were aspirated, the glycerol phase containing lysed erythrocytes was prepared for K^+ measurement using atomic absorption spectroscopy (Perkin-Elmer model 560).

The amount of medium carried with the cells as they moved through the oil into the glycerol was determined for parallel replicates of cell suspension by the method of Freel *et al.* (1973) using ^{14}C-PEG as an extracellular space marker. The K^+ content of the trapped medium (usually about 1% of the total cell pellet K^+) was subtracted from the K^+ content of the glycerinated cell pellet to yield intracellular K^+.

Intracellular Na^+ was measured using the same technique described above for K^+, except that the trapped volume was measured directly for each sample instead of for parallel replicates. Since ASW Na^+ concentrations are high, the Na^+ level of the trapped medium could account for up to 60% of total cell pellet Na^+. After the cells had been sampled as described above, the medium and oil layers were aspirated, and an additional 0.1 ml of 25% glycerol was added to the cell pellet. This glycerol suspension was homogenized, and 0.05 ml was removed for trapped volume estimation. The remaining 0.15 ml was used to measure cellular Na^+ content by atomic absorption spectroscopy.

The technique for determining intracellular Cl^- was identical to that used for Na^+, except that Cl^- was measured by amperometric titration (Chloridometer, Buchler-Cotlove).

Statistical differences in intracellular K^+, Na^+, and Cl^- content were determined using analysis of variance and the Student-Newman Kuels multiple range test.

Results

Noetia erythrocytes swell rapidly in hypoosmotic ASW, but within 10 min ($P \leq 0.05$) after hypoosmotic exposure they begin to recover towards their original volume (Fig. 1). Although volume recovery continues for at least two hours after hypoosmotic exposure, very close to half of the volume decrease happens within 20 min of hypoosmotic exposure.

Taurine efflux from *Noetia* erythrocytes in hypoosmotic media increases significantly during the volume regulatory period. However, most of the taurine efflux occurs between 10 and 60 min of hypoosmotic exposure (Fig. 2).

There is no significant difference between the Na^+ content of isosmotic and hypoosmotic cells over the duration of the two hour time course (Fig. 3a). However, intracellular K^+ decreases by about 15%, from 98 to 85 nmoles/10^6 cells within 10 min

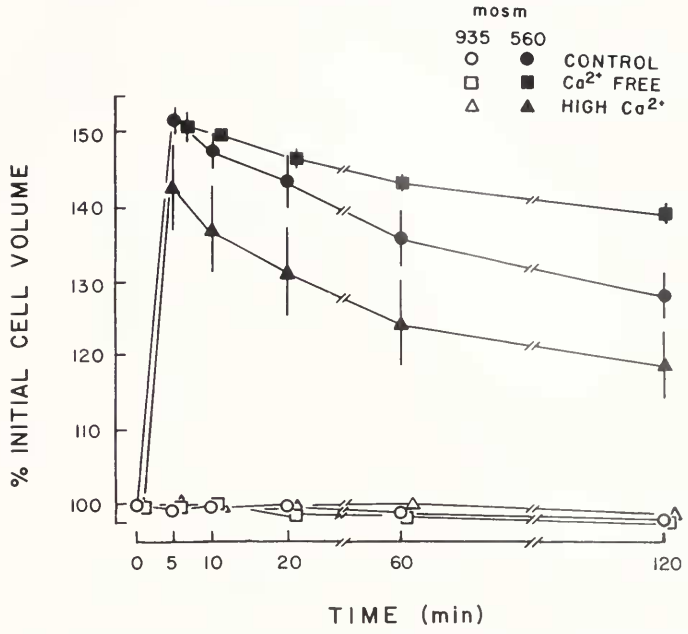

FIGURE 1. Volume changes of *Noetia ponderosa* erythrocytes exposed to isosmotic (935 mosm) or hypoosmotic (560 mosm) ASW of varying Ca^{2+} concentration. Vertical bars are ±1 standard error of the mean (S.E.M.).

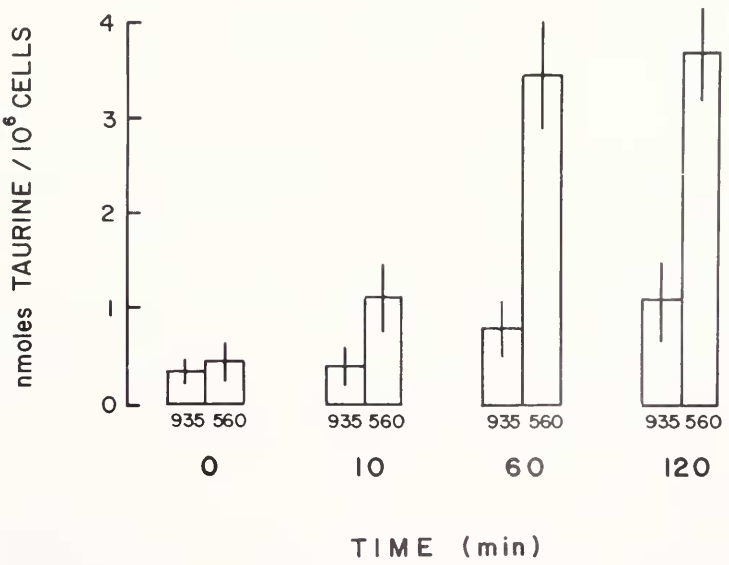

FIGURE 2. Taurine efflux from *Noetia ponderosa* erythrocytes in isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ±1 S.E.M.

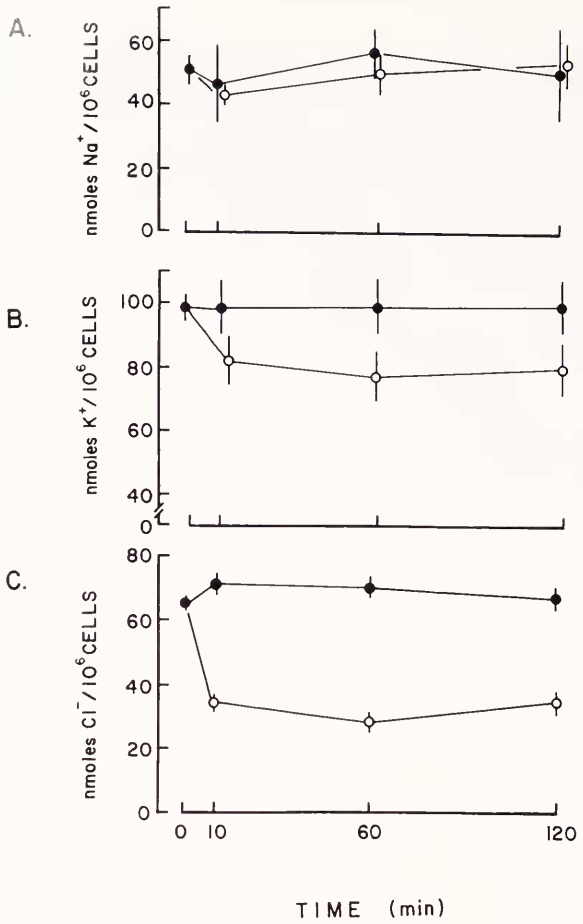

FIGURE 3. Intracellular Na^+ (A), K^+ (B), and Cl^- (C) content of *Noetia ponderosa* erythrocytes in isosmotic (closed circles) and hypoosmotic (open circles) ASW. Vertical bars are ±1 S.E.M.

after hypoosmotic exposure, and little further decrease occurs subsequently (Fig. 3b). In addition, intracellular Cl^- decreases by 50% within 10 min of hypoosmotic exposure, from about 70 to 35 nmoles/10^6 cells (Fig. 3c). Thus, K^+ and Cl^-, but not Na^+, leave *Noetia* erythrocytes after hypoosmotic exposure, and in contrast with the taurine efflux, this movement of intracellular ions begins immediately. The Cl^- efflux from *Noetia* erythrocytes was only partially balanced by the K^+ loss. Thus, other cations besides K^+ (but not Na^+) must leave the clam erythrocyte during the early phase of volume recovery.

In the absence of Ca^{2+}, the volume regulation in response to hypoosmotic ASW is partially inhibited (Fig. 1). Cells in the hypoosmotic Ca^{2+}-free medium recovered slightly less than half the volume of control cells in normal hypoosmotic medium. Ca^{2+} lack did not affect the volume of erythrocytes in isosmotic ASW (Fig. 1).

Isosmotic Ca^{2+}-free ASW had no significant effect on cellular K^+ (Fig. 4a). Moreover, the usual hypoosmotic effect on cellular K^+ content occurred whether or not Ca^{2+} was present in the medium (Fig. 4a). The effect of Ca^{2+} lack on intracellular Cl^- content was similar in pattern to that of K^+. The Cl^- content of the cells was

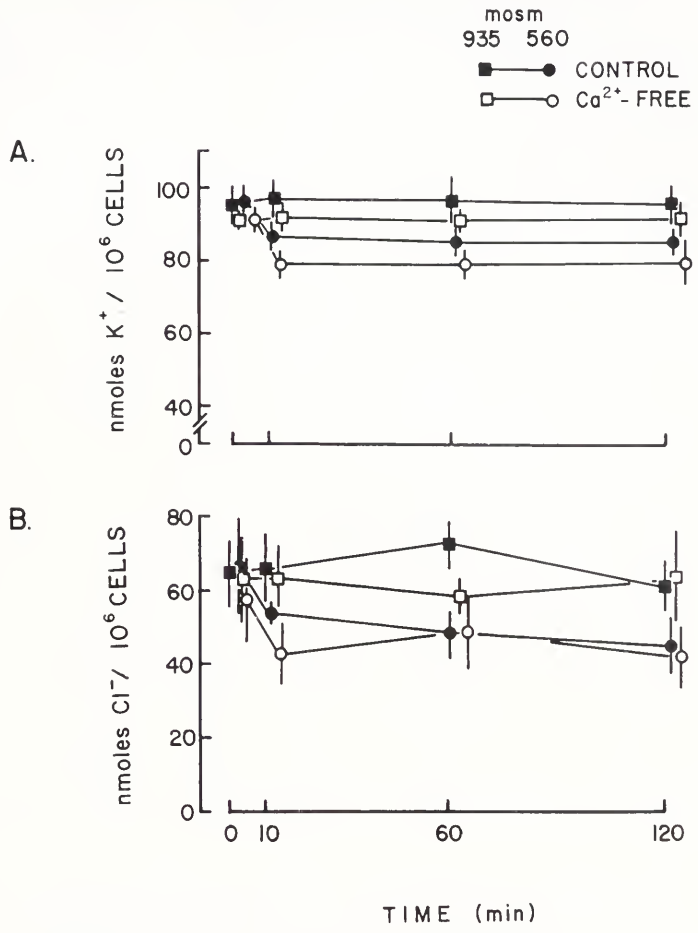

FIGURE 4. Intracellular K^+ (A) and Cl^- (B) content of *Noetia ponderosa* erythrocytes in control and Ca^{2+}-free isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ±1 S.E.M.

altered only by the usual amounts in response to the osmotic stress, regardless of $[Ca^{2+}]_0$ (Fig. 4b).

In contrast, taurine efflux from *Noetia* erythrocytes in Ca^{2+}-free hypoosmotic ASW was significantly less than that of cells in hypoosmotic ASW after 60 and 120 min (Fig. 5). A small yet significant efflux of taurine occurred from clam erythrocytes in Ca^{2+}-free isosmotic ASW (Fig. 5). Therefore, taurine efflux was inhibited in Ca^{2+}-free hypoosmotic ASW, while K^+ and Cl^- effluxes were unchanged.

Cell volume regulation by clam erythrocytes in media containing elevated Ca^{2+} was potentiated (Fig. 1). Hypoosmotically stressed clam erythrocytes recovered to 119% of initial cell volume in the presence of high Ca^{2+}, while the cells in hypoosmotic medium containing normal Ca^{2+} recovered to only 129% of initial cell volume. No significant change in erythrocyte volume occurred upon exposure of the cells to isosmotic high Ca^{2+} ASW.

High Ca^{2+} had no effect on taurine efflux from clam erythrocytes in isosmotic ASW (Fig. 7) or on erythrocyte K^+ or Cl^- content in either hypoosmotic or isosmotic media (Fig. 6a, b). The usual decrease in intracellular K^+ and Cl^- within 10 min

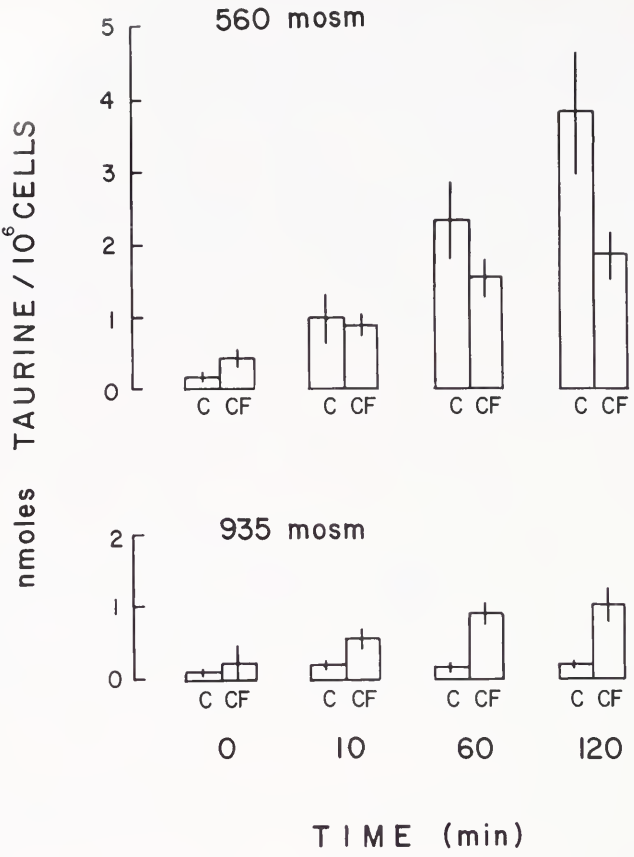

FIGURE 5. Taurine efflux from *Noetia ponderosa* erythrocytes in normal (C) and Ca^{2+}-free (CF) isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ±1 S.E.M.

of exposure to hypoosmotic media (about 10 and 30 nmoles/10^6 cells, respectively) occurred in the presence of high Ca^{2+} (Fig. 6a, b). In contrast, the taurine efflux from *Noetia* erythrocytes in high Ca^{2+} hypoosmotic media was significantly greater, almost twice that of control cells (Fig. 7).

Thus, both Ca^{2+}-free hypoosmotic ASW and high-Ca^{2+} hypoosmotic ASW affect the volume regulation and taurine efflux, but not K^+ or Cl^- efflux, from clam erythrocytes.

DISCUSSION

Our results confirm our earlier report (Smith and Pierce, 1983) that cell volume regulation by *Noetia* erythrocytes in response to hypoosmotic conditions results from the efflux of both organic and inorganic osmolytes. *Noetia* erythrocytes use effluxes of K^+, Cl^-, and amino acids to regulate cell volume during a hypoosmotic stress. The ion efflux occurs immediately in response to the stress while the efflux of taurine is delayed. Both cell volume regulation and taurine efflux are dependent on extracellular [Ca^{2+}] but the ion efflux is not. Therefore, a mechanism is present which allows the two independent membrane permeability systems to produce a coordinated efflux of organic and inorganic osmolytes from *Noetia* erythrocytes. Both K^+ and Cl^- leave

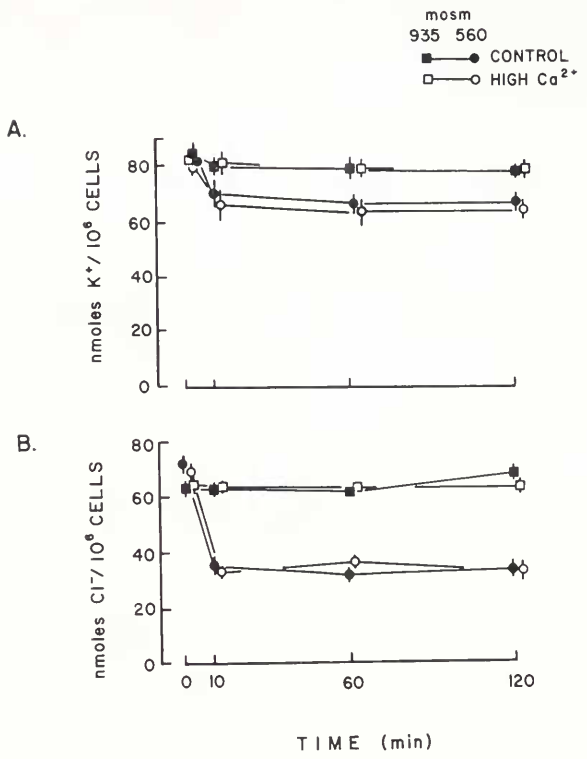

FIGURE 6. *Noetia ponderosa* erythrocyte intracellular K^+ (A) and Cl^- (B) content in control and high Ca^{2+} isosmotic (150 m*M*, 935 mosm) and hypoosmotic (90 m*M*, 560 mosm) ASW. Vertical bars are ±1 S.E.M.

the *Noetia* erythrocytes immediately after the salinity stress while taurine concentrations do not change for many minutes. Thus, the two ions act alone as the osmolytes during the initial period of volume recovery. The amount of volume recovery resulting from the ionic efflux may be substantial. We do not know the maximum volume attained by the *Noetia* cells, but it is likely that volume recovery is well underway at our first sampling interval (5 min) (see, for example, Costa *et al.*, 1980). Therefore, the initial stage of ionic efflux accounts for most of the volume recovery.

The significance of a dual osmolyte efflux system is not yet clear, although it is not uncommon (Vislie, 1980; Costa and Pierce, 1983; Warren and Pierce, 1983; Moran and Pierce, 1984; Hoffman *et al.*, 1984). The differences between the ionic and organic osmolyte effluxes may provide a clue. Although the inorganic osmolyte efflux from the *Noetia* cells occurred immediately in response to hypoosmotic exposure, the concentrations of the ions may not remain reduced. In at least one case, after the initial reduction, the intracellular ion concentration was partially restored as the organic components were reduced (Warren, 1982). On the other hand, while the efflux of organic osmolytes is initiated later in the response, no restoration of pre-stress concentrations occurs. Although the data are limited, they suggest that the organic osmolytes are not functioning as effectors in direct response to the osmotic stress, but rather as osmotic replacements as the ions are regulated back to homeostatic levels. The organic osmolytes are kept at levels which produce cellular osmotic stabilization without the detrimental effects on cell functions that might be caused

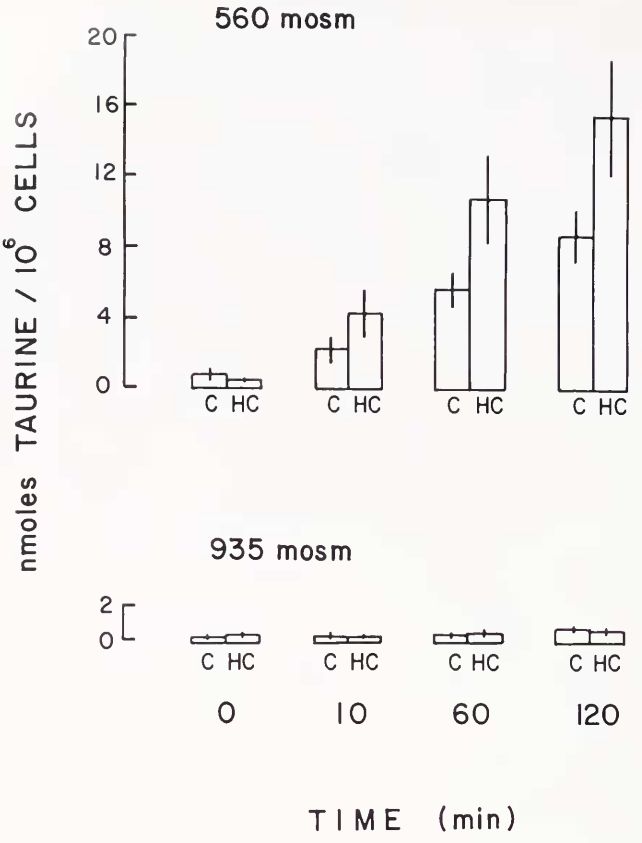

FIGURE 7. Taurine efflux from *Noetia ponderosa* erythrocytes in control (C) and increased Ca^{2+} (HC) isosmotic (150 m*M*, 935 mosm) and hypoosmotic (90 m*M*, 560 mosm) ASW. Vertical bars are ±1 S.E.M.

by marked reduction in inorganic ion concentration. We are presently evaluating these ideas experimentally.

Our results indicate that the *Noetia* erythrocyte displays the components of volume regulation found among many other cell types. Rapid decreases of intracellular K^+ following hypoosmotic treatment occur in other invertebrate (Kevers *et al.*, 1979a, b, 1981; Costa and Pierce, 1983; Moran and Pierce, 1984) and vertebrate (Kregenow, 1971; Cala, 1977; Cheung *et al.*, 1982) cells. A hypoosmotically induced Cl^- efflux concomitant with K^+ release occurs in crustacean axons (Kevers *et al.*, 1979b) and teleost erythrocytes (Lauf, 1982). Cl^- loss is also associated with Na^+ efflux in *Limulus* myocardium (Warren and Pierce, 1982) and rat liver (van Rossum and Russo, 1984). Thus, the initial ionically based stage of hypoosmotic volume regulation in *Noetia* cells is qualitatively similar to the responses of many other cell types.

The hypoosmotically induced Na^+ and Cl^- effluxes from *Limulus* myocardium, like the ionic effluxes from *Noetia* blood cells, are unaffected by the absence of Ca^{2+} (and Mg^{2+}) (Warren, 1982). However, the ionic components of the volume regulating systems of some other cell types are sensitive to the ambient level of Ca^{2+}. For instance, the K^+ efflux from *Glycera* coelomocytes exposed to either isosmotic or hypoosmotic Ca^{2+}-free media is potentiated (Costa and Pierce, 1983). External Ca^{2+} is

important in the regulation of K^+ efflux from vertebrate cells as well. For example, K^+ efflux from amphibian red blood cells was inhibited in Ca^{2+}-free hypoosmotic media (Cala, 1983). Likewise, K^+ efflux and volume regulatory ability were impaired in Ehrlich ascites tumor cells (Hoffman *et al.*, 1984) and human lymphocytes (Grinstein, 1983) by Ca^{2+}-free conditions.

Both volume regulation and taurine efflux are inhibited in *Noetia* erythrocytes exposed to Ca^{2+}-free hypoosmotic ASW, and both are potentiated in high Ca^{2+} hypoosmotic ASW. Similarly, cell volume regulation is inhibited by Ca^{2+}-free hypoosmotic media in *Callinectes* axons (Gerard, 1975) and *Glycera* coelomocytes, although the FAA efflux from *Glycera* coelomocytes in divalent cation-free hypoosmotic media is potentiated (Costa and Pierce, 1983). In summary, the Ca^{2+} effects on volume recovery and the effluxes underlying it are not the same in every cell type. Ionic or organic osmolytes may be effected by Ca^{2+} change, but in the *Noetia* cells only the amino acid portion of the response is Ca^{2+} sensitive.

An additional aspect of Ca^{2+} regulation is suggested by the small amount of taurine released from *Noetia* erythrocytes in the Ca^{2+}-free isosmotic medium. A similar leak of amino acids occurred from *Glycera* coelomocytes in divalent cation-free isosmotic media (Costa and Pierce, 1983) and mussel ventricles (Pierce and Greenberg, 1973). The increase in FAA permeability in Ca^{2+}-free isosmotic media, in contrast to its decrease in hypoosmotic media, suggests that different Ca^{2+} sensitive mechanisms exist for the control of membrane permeability at normal and lowered osmotic concentrations. The increased membrane permeability to taurine caused by Ca^{2+}-free isosmotic media may be due to a lack of divalent cations that bind to and stabilize membranes (Lin and Macey, 1978; Papahadjopolous, 1978; Swinehart *et al.*, 1980). But in any case, Ca^{2+} may have more than one action on osmolyte permeability depending upon the osmotic environment.

In conclusion, the characteristics of the volume response of the *Noetia* cells and their similarity to those of other cell types suggests that the clam red blood cell will be an excellent model with which to examine further effects and the roles of Ca^{2+} in the regulation of cell volume.

Acknowledgments

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LITERATURE CITED

Amende, L. M., and S. K. Pierce. 1980. Cellular volume regulation in salinity stressed molluscs. The response of *Noetia ponderosa* (Arcidae) red blood cells to osmotic variation. *J. Comp. Physiol.* **138:** 283–289.

Cala, P. M. 1977. Volume regulation by flounder red blood cells in anisotonic media. *J. Gen. Physiol.* **69:** 537–552.

Cala, P. M. 1983. Cell volume regulation by *Amphiuma* red blood cells. The role of Ca^{2+} as a modulator of alkali metal/H^+ exchange. *J. Gen. Physiol.* **82:** 761–784.

Cheung, R. K., S. Grinstein, H. M. Dosch, and E. W. Gelfand. 1982. Volume regulation by human lymphocytes: characterization of the ionic basis for regulatory decrease. *J. Comp. Physiol.* **112:** 189–196.

Costa, C. J., S. K. Pierce, and M. K. Warren. 1980. The intracellular mechanism of salinity tolerance

in polychaetes: volume regulation by isolated *Glycera dibranchiata* red coelomocytes. *Biol. Bull.* **159:** 626–638.

COSTA, C. J., AND S. K. PIERCE. 1983. Volume regulation in the red coelomocytes of *Glycera dibranchiata:* an interaction of amino acid and K^+ effluxes. *J. Comp. Physiol.* **151:** 133–144.

FREEL, R. W., S. G. MEDLER, AND M. E. CLARK. 1973. Solute adjustments in the coelomic fluid and muscle fibers of a euryhaline polychaete, *Neanthes succinea* adapted to various salinities. *Biol. Bull.* **144:** 289–303.

GERARD, J. F. 1975. Volume regulation and alanine transport. Response of isolated axons of *Callinectes sapidus* Rathbun to hypo-osmotic conditions. *Comp. Physiol. Biochem.* **51A:** 225–229.

GRINSTEIN, S., S. COHEN, B. SARKADI, AND A. ROTHSTEIN. 1983. Induction of ^{86}Rb fluxes by Ca^{2+} and volume changes in thymocytes and their isolated membranes. *J. Cell Physiol.* **116:** 352–362.

HOFFMAN, E. K., L. O. SIMONSEN, AND I. H. LAMBERT. 1984. Volume-induced increase of K^+ and Cl^- permeabilities in Ehrlich ascites tumor cells. Role of Ca^{2+}. *J. Membrane Biol.* **78:** 211–222.

KEVERS, C., A. PEQUEX, AND R. GILLES. 1979a. Effects of hypo- and hyperosmotic shocks on the volume and ion contents of *Carcinus meanus* isolated axons. *Comp. Biochem. Physiol.* **64A:** 427–431.

KEVERS, C., A. PEQUEX, AND R. GILLES. 1979b. Effects of an hypo-osmotic shock on Na^+, K^+, and Cl^- levels in isolated axons of *Carcinus meanus*. *J. Comp. Physiol.* **129:** 365–371.

KEVERS, C., A. PEQUEX, AND R. GILLES. 1981. Role of K^+ in the cell volume regulation response of isolated axons of *Carcinus meanus* submitted to hypo-osmotic conditions. *Molec. Physiol.* **1:** 13–22.

KREGENOW, F. M. 1971. The response of duck erythrocytes to non-hemolytic media. *J. Gen. Physiol.* **58:** 372–412.

LAUF, P. K. 1982. Evidence for chloride dependent potassium and water transport induced by hypoosmotic stress. *J. Comp. Physiol.* **146:** 9–16.

LIN, G. S. B., AND R. I. MACEY. 1978. Shape and stability changes in human erythrocyte membranes induced by metal cations. *Biochim. Biophys. Acta* **512:** 270–283.

MORAN, W. M., AND S. K. PIERCE. 1984. The mechanism of crustacean salinity tolerance: cell volume regulation by K^+ and glycine effluxes. *Mar. Biol.* **81:** 41–46.

PAPAHADJOPOLOUS, D. 1978. Role of ionic environment on self-assembly properties of phospholipid membranes. Pp. 77–91 in *Light Transducing Membranes*, D. W. Deamer, ed. Academic Press, New York.

PIERCE, S. K. 1982. Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. *Biol. Bull.* **163:** 405–419.

PIERCE, S. K., AND L. M. AMENDE. 1981. Control mechanisms of amino acid mediated cell volume regulation in salinity stressed molluscs. *J. Exp. Zool.* **215:** 247–257.

PIERCE, S. K., S. C. EDWARDS, P. M. MAZZOCCHI, L. J. KLINGER, AND M. K. WARREN. 1984. Proline betaine: a unique osmolyte in an extremely euryhaline osmoconformer. *Biol. Bull.* **167:** 495–500.

PIERCE, S. K., AND M. J. GREENBERG. 1973. The initiation and control of free amino acid regulation of cell volume regulation in salinity stressed marine bivalves. *J. Exp. Biol.* **59:** 435–440.

VAN ROSSUM, G. D. V., AND M. A. RUSSO. 1984. Requirement of Cl and Na^+ for the ouabain-resistant control of cell volume in slices of rat liver. *J. Membrane Biol.* **77:** 63–76.

SMITH, L. H., AND S. K. PIERCE. 1983. *Noetia ponderosa* erythrocytes use intracellular ions for initial volume regulation during hypoosmotic stress. *Am. Zool.* **23:** 994.

STEELE, R. G. D., AND J. H. TORRIE. 1960. *Principles and Procedures of Statistics*. McGraw-Hill, New York.

SWINEHART, J. H., J. H. CROWE, A. P. GIANNINI, AND D. A. ROSENBAUM. 1980. Effects of divalent ion concentration on amino acid and divalent cation fluxes in gills of the bivalve mollusc, *Mytilus californianus*. *J. Exp. Zool.* **212:** 389–396.

VISLIE, T. 1980. Cell volume regulation in isolated perfused heart ventricle of the flounder (*Platichthys flesus*). *Comp. Biochem. Physiol.* **65A:** 19–27.

WARREN, M. K. 1982. Two cell volume regulatory systems in the *Limulus* myocardium: an interaction of ions and quaternary ammonium compounds. Ph.D. thesis. University of Maryland, College Park. 58 pp.

WARREN, M. K., AND S. K. PIERCE. 1982. Two cell volume regulatory systems in the *Limulus* myocardium: an interaction of quarternary ammonium compounds and ions. *Biol. Bull.* **163:** 504–516.